825. *Transamination. Part II.* The Non-enzymic Reactions between Pyridoxamine and Pyruvic Acid and between Pyridoxal and Alanine.*

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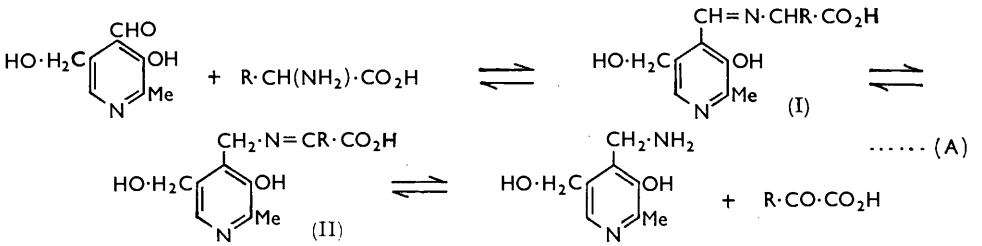
The reaction between pyridoxamine and pyruvic acid in water at 25° over the range pH 7—10 yields pyridoxal and alanine. Kinetic and spectroscopic studies show that the reaction proceeds by the fast and reversible formation of the Schiff's base. The rate-limiting step, which is general-acid catalysed, is the interconversion of this and the isomeric Schiff's base, *i.e.*, the change ·CH=N·CHMe·CO_2H ⇌ ·CH_2·N=CMe·CO_2H. The reverse reaction (formation of pyridoxamine and pyruvic acid) has also been studied, but in less detail. The mechanism of the reactions is discussed.

SNELL and his co-workers [1] have shown that many of the enzymic reactions in which pyridoxal 5′-phosphate is involved as co-factor can be simulated *in vitro* by heating a mixture of an appropriate amino-acid, pyridoxal, and a metal-ion catalyst. Following earlier suggestions [2] that imine intermediates are involved, Snell and his co-workers [3] suggested that the transamination proceeds essentially as shown in scheme (A) (which for simplicity has been written without reference to ionic states or to the formation of chelates between the intermediates and any metal ions present in solution).



Intermediates of type (I) and their metal chelates (C_I) have been isolated.[4] Semi-quantitative kinetic studies [5] support the view that under Snell's conditions reaction proceeds *via* metal chelates C_I and C_{II}. Metzler [6] showed that in aqueous solution, imines

* Part I, *J.*, 1961, 1698.

[1] Snell, *J. Amer. Chem. Soc.*, 1945, **67**, 194; Snell and Metzler, *J. Amer. Chem. Soc.*, 1952, **74**, 979; Metzler, Olivard, and Snell, *J. Amer. Chem. Soc.*, 1954, **76**, 644.

[2] Braunstein, *Enzymologia*, 1937, **2**, 138; Herbst, *Adv. Enzymology*, 1944, **4**, 75; Braunstein and Shemyakin, *Biokhimiya*, 1953, **18**, 393.

[3] Metzler, Ikawa, and Snell, *J. Amer. Chem. Soc.*, 1954, **76**, 648.

[4] Hamis, Heyl, and Folkers, *J. Biol. Chem.*, 1944, **154**, 315; Christensen and Collins, *ibid.*, 1956, **220**, 279; Heyl, Luz, Harris, and Folkers, *J. Amer. Chem. Soc.*, 1948, **70**, 3429, 3669; 1952, **74**, 414; Christensen, *ibid.*, 1957, **79**, 4073.

[5] Fasella, Lis, Siliprandi, and Baglioni, *Biochim. Biophys. Acta*, 1957, **23**, 417.

[6] Metzler, *J. Amer. Chem. Soc.*, 1957, **79**, 485.

(*e.g.*, I) are rapidly and reversibly formed from pyridoxal and amino-acids, and the equilibrium constants involved were measured spectrophotometrically. Matsuo [7] found that similar reactions occur with pyridoxal 5'-phosphate in ethanol and that, under these conditions, transamination occurs readily even in the absence of metal-ion catalysts.

This paper reports a detailed kinetic study of the reaction:

$$\text{Pyridoxamine} + \text{Pyruvate} \rightleftharpoons \text{Pyridoxal} + \text{Alanine} \quad . \quad . \quad . \quad \text{(B)}$$

Experimental

Materials.—Pyridoxamine hydrate crystallised from an aqueous solution of pyridoxamine dihydrochloride which had been neutralised with sodium hydroxide. It was washed with water and methanol and twice recrystallised, under nitrogen, from water (Found: C, 47·4; H, 8·1; H_2O, 16·6. Calc. for $C_8H_{16}N_2O_4$: C, 47·0; H, 8·4; H_2O, 17·5%). The dissociation constants $pK_{a,1}$, $pK_{a,2}$, $pK_{a,3}$, determined by titration with a glass electrode, were 3·33, 8·08, and 10·33, respectively.

Pyridoxal ethyl acetal hydrochloride (Roche Products Ltd.) was dissolved in water and heated at 60°, for 10 min.; pyridoxal separated after addition of an equivalent of sodium hydrogen carbonate and was recrystallised, under nitrogen, from water (Found: C, 57·2; H, 5·7. Calc. for $C_8H_7NO_3$: C, 57·5; H, 5·4%).

DL-Alanine (B.D.H.), when recrystallised from aqueous ethanol, was chromatographically homogeneous.

Commercial samples of *N*-methyldiethanolamine, *NN*-dimethylethanolamine, and triethanolamine were distilled under reduced pressure. Values of pK_a were 8·61, 9·33, and 8·00 respectively.

NN-Dimethylglycine hydrochloride was a commercial sample (Found: equiv., 140·4. Calc. for $C_4H_{10}ClNO_2$: equiv., 139·6), pK_a 9·70.

Potassium pyruvate was obtained by adding freshly distilled pyruvic acid, b. p. 59—61°/10 mm., to an equivalent of potassium ethoxide in anhydrous ethanol. The salt was filtered off, washed with cold ethanol and then ether, dried, and recrystallised by addition of ethanol to a concentrated aqueous solution.

All inorganic reagents were of " AnalaR " grade.

Preparations.—Potassium α-4-pyridoxylideneaminopropionate * (see Heyl *et al.*[4]) was prepared by mixing equivalent amounts (1 mmole) of pyridoxal and the potassium salt of alanine in anhydrous ethanol (5 ml.). Recrystallisation from anhydrous ethanol gave yellow crystals which were exceedingly deliquescent and were therefore handled in a dry box (Found: C, 46·9; H, 5·5; N, 9·1. Calc. for $C_{11}H_{13}KN_2O_4$: C, 47·8; H, 4·7; N, 10·1%). In aqueous solution (pH 6·9, 10·5; or 0·1M-HCl) hydrolysis is very rapid, the ultraviolet absorption spectrum becoming identical with that of an equimolar solution of pyridoxal. In anhydrous ethanol maxima occur at 256, 337, 420 mμ (ε 7150, 3340, 2030, respectively) (cf. pyridoxal, 282, 325 mμ; ε 5020, 780 respectively).

Nickel di-(α-4-pyridoxylideneaminopropionate) was obtained at 0° as brown crystals from an aqueous solution (4 ml.) containing nickel acetate (0·5 mmole), pyridoxal (1 mmole), the potassium salt of alanine (1 mmole), and sodium acetate (0·5 mmole). Analysis was by colorimetric determination of nickel [8] after digestion of the complex with nitric–sulphuric acid, or by spectrophotometric determination of pyridoxal after complete hydrolysis in 0·1M-hydrochloric acid (pyridoxal, 288 mμ, ε 8840; pyridoxamine, 293 mμ, ε 8530, isosbestic point 291·5 mμ, ε 8460; all in 0·1M-HCl) (Found: Ni, 9·7; pyridoxal, 54·8; after exhaustive drying, Ni, 9·9; pyridoxal, 56·6. $C_{24}H_{30}N_4NiO_{10},H_2O$ requires Ni, 9·6; pyridoxal, 54·6. $C_{24}H_{30}N_4NiO_{10}$ requires Ni, 9·9; pyridoxal, 56·4%). The magnetic moment, calculated from the paramagnetic gain of the solid was found to be 3·45 B.M. (*M* 610·7). The ultraviolet absorption spectrum in water is similar to that of pyridoxal (max. at 252 and 315 mμ) with an additional peak at 382 mμ (ε 5600). At pH 6·9 and 25°, hydrolysis in 2×10^{-4}M-solution occurs by a first-order process ($t_{\frac{1}{2}} \sim 22$ min.), giving Ni^{II} ions, pyridoxal, and alanine, the last two being identified chromatographically.

Nickel di-(α-4-pyridoxyliminopropionate) separated as green crystals from a cold solution of

* In this nomenclature, the numeral (here 4) refers to the *side chain* that is involved.

[7] Matsuo, *J. Amer. Chem. Soc.*, 1957, **79**, 2011, 2016.

[8] Mitchell and Mellon, *Analyt. Chem.*, 1945, **17**, 380.

pyridoxamine (2 mmole), potassium pyruvate (2 mmole), and nickel acetate (1 mmole) in anhydrous ethanol (50 ml.). It was dried *in vacuo* over P_2O_5 (Found: pyridoxamine, 58·3; Ni, 11·6; N, 10·5. $C_{22}H_{26}N_4NiO_8$ requires pyridoxamine, 57·8; N, 11·0; N, 10·5%) (spectrophotometric analysis for pyridoxamine was made after total hydrolysis in 0·1M-HCl). The magnetic moment was 3·32 B.M. Hydrolysis was rapid in dilute solution, giving Ni^{II} ions, pyridoxamine, and pyruvate, the last two identified chromatographically. At pH 6·9 and 25°, concentrated solutions deposit the isomeric nickel salt.

Chromatography.—Pyridoxal, pyridoxamine, and alanine were identified by using the following solvents (proportions by volume). (1) The top layer from 1 : 1 butan-1-ol–5% acetic acid. (2) The top layer from 1 : 1 butan-1-ol–5% aqueous ammonia. (3) Propan-1-ol–water–aqueous ammonia hydroxide (*d* 0·880) (85 : 10 : 5). Spots were developed as follows: pyridoxal, yellow on exposure to ammonia or spraying with 10% ethanolamine solution; pyridoxamine, tangerine-coloured with ninhydrin (detected on single application of a 0·01M-solution); alanine, blue-violet with ninhydrin spray.

R_F values were: pyridoxal 0·48—0·50 and 0·25 for solvents 1 and 2, respectively; pyridoxamine 0·12 and 0·44—0·50 (elongated) for solvents 1 and 3, respectively; alanine 0·09 and 0·18—0·21 for solvents 1 and 3, respectively.

The solvent used for pyruvic acid 2,4-dinitrophenylhydrazone was butan-1-ol–ethanol–0·5M-ammonia (7 : 10 : 2), in which R_F values were: *trans* 0·58—0·60, *cis*-0·71—0·73. The *trans*-isomer gave an intense brown colour on spraying with 20% aqueous sodium hydroxide.

Kinetic Methods.—Attention to the recorded detail is essential.

Reaction between pyridoxamine and pyruvate. The reactions were followed by estimating pyridoxal as described by Snell and Metzler.[1] The optical density (362·5 mμ, ε 6740) of the final solution in aqueous ethanolamine (25% by volume) was constant for 1 hr. and unaffected by temperature (18—27°), the presence of pyridoxamine (0·002M) or pyruvate (0·001—0·5M), or any of the buffers used.

Buffer solutions containing nitrogen bases were made either by adding the appropriate amount of hydrochloric acid to the base or by adding the appropriate amount of carbon dioxide-free sodium hydroxide solution to the hydrochloride.

Pyridoxamine was used as the dihydrochloride. An amount of carbon dioxide-free sodium hydroxide solution (calculated from the pK_a's of pyridoxamine) sufficient to bring the solution of pyridoxamine dihydrochloride to the same pH as the buffer was, therefore, included in each reaction mixture.

Potassium pyruvate had a negligible effect on the pH of solutions containing 0·1M-buffer over the range pH 7—10. At lower buffer concentrations, it was necessary, particularly at high pyruvate levels, to adjust the pH to the required value after addition of the potassium pyruvate. At the end of each kinetic run the pH of the solution was usually determined with a glass micro-electrode. No significant differences between expected and measured values were observed.

Ionic strength (μ) was controlled, where desired, by addition of sodium chloride, and was then calculated from the concentrations of pyruvate ion, sodium chloride and ionic forms of the buffer and pyridoxamine.

The following details of a kinetic run with 0·02M-pyridoxamine and 0·30M-pyruvate at pH 9·00 and 25° are illustrative: a buffer solution (6·25 ml.), obtained by mixing equal volumes of solutions of *NN*-dimethylglycine hydrochloride (0·4M) and sodium hydroxide (0·612M), was added to potassium pyruvate (0·4769 g.) and made up to 10 ml. with distilled water. A portion (8 ml.) was allowed to come to thermal equilibrium in a thermostat bath (25°). The reaction was started by adding a 0·1M-solution (2 ml.), at 25°, of pyridoxamine dihydrochloride. Aliquot parts (1 ml.) were removed at intervals and run into 1 : 1 v/v aqueous ethanolamine. The solutions were made up to 10 ml. and the optical densities (at 362·5 mμ) measured with a Unicam photoelectric spectrophotometer (1·0 cm. (silica cells), with a 25% ethanolamine solution in the control cell. At the end of the experiment the residual solution had pH 8·98 (glass electrode, standardised at pH 6·86). The results were as follows:

Time (min.)	5	15	25	35	45	55
Optical density (0·0)	0·110	0·254	0·397	0·540	0·682	0·820

The final point represents *ca.* 6·1% reaction and up to this point the change of optical density is linear with time. The initial rate is, therefore, 0·00237 O.D. unit sec.$^{-1}$, *i.e.*, 3·51 ×

10^{-7} mole $l.^{-1}$ $sec.^{-1}$. For convenience and in order to avoid complications due to imine formation between pyridoxal and pyridoxamine the reactions were usually followed for only 5% of the total reaction. Rates of formation of pyridoxal rather than rate coefficients were, therefore, usually calculated. Runs in which much greater proportions of the reaction were followed showed the expected first-order dependence of rate on pyridoxamine concentration (pyridoxal in large excess). Chromatography of solutions similar to that detailed above showed the presence of pyridoxal and alanine in amounts which increased with reaction time.

Reaction between pyridoxal and alanine. (*a*) Estimation of pyruvate (10^{-3} to 10^{-4}M) in the presence of pyridoxal (10^{-2}M). Addition of 2,4-dinitrophenylhydrazine gave a solution containing the dinitrophenylhydrazones of pyruvic acid and of pyridoxal and unchanged reagent. From strongly acidic solution, chloroform extracts only the pyruvic acid derivative. The detailed procedure was as follows: The solution (1·00 ml.) was mixed with the dinitrophenylhydrazine (2 ml. of a solution containing 50 mg. of recrystallised material, 1·5 ml. of 72% perchloric acid and 14·5 ml. of water); water (4 ml.) was added and the solution set aside for 5 min. After crystallisation of the pyridoxal derivative (induced, if necessary, by scratching), the solution was left for a further 15 min. and then filtered through a cotton-wool plug. An aliquot part (4 ml.) was acidified with 72% perchloric acid (1 ml.) and extracted with chloroform (5 ml., then 3 × 1 ml.). The combined chloroform extracts were shaken with borate buffer (5 ml.; 0·5M; pH 9·2) [which was then washed with chloroform (1 ml.) and centrifuged. The optical density at 372·5 mμ (1 cm. silica cells) was then measured against a water control with a Unicam photoelectric spectrophotometer. By using potassium pyruvate solutions 0·1M in pyridoxal, pyruvic acid dinitrophenylhydrazone was found to have ε 22,200, compared with ε 22,900 for the pure *trans*-derivative (obtained by repeated crystallisation from ethyl acetate) added directly to borate buffer of pH 9·2.

By the above procedure, the yield of pyridoxal dinitrophenylhydrazone is only *ca.* 90%. The unchanged pyridoxal does not, however, interfere since it is not extracted by chloroform. If the original solution is left for longer than 15 min. some 2,4-dinitrophenol is formed [identified chromatographically; butan-1-ol–ethanol–0·5M-NH_3 (7 : 1 : 2); R_F 0·71—0·73], and this accompanies the pyruvic acid dinitrophenylhydrazone in the separation. This complication can only be avoided by use of freshly prepared dinitrophenylhydrazine reagent and by adherence to the above stated times.

The following details of a kinetic experiment (25°, pH 10·0, alanine 0·20M, pyridoxal 0·01M) are illustrative. A solution (5 ml.) 0·40M in alanine and 0·252M in sodium hydroxide was allowed to come to temperature equilibrium. The reaction was started by adding a 0·2M-solution (5 ml.) of pyridoxal, also at 25°. Aliquot parts (1 ml.) were removed at appropriate times and run into the dinitrophenylhydrazine reagent (2 ml.). The results were:

Time (min.)	5	25	45	65	85	105
Optical density (372·5 mμ) ...	0·092	0·218	0·340	0·479	0·598	0·720

The rate of change of optical density is therefore 0·00632 unit $min.^{-1}$: hence the initial reaction rate is $7·5 \times 0·00632/(60 \times 22,200) = 3·55 \times 10^{-8}$ mole (of pyruvate) $l.^{-1}$ $sec.^{-1}$. The final solutions obtained in a similar experiment were acidified and extracted with chloroform. Chromatography gave two spots with R_F values identical with those of the isomeric pyruvic acid 2,4-dinitrophenylhydrazones.

(*b*) Estimation of pyridoxamine. Pyridoxamine was estimated spectrophotometrically after removal of pyruvic acid (present in small amounts) and pyridoxal (present in large amounts) as their dinitrophenylhydrazones. Extraction with chloroform from acidic solution removed the former (and any 2,4-dinitrophenol); further extraction at pH 7 removed the latter. The detailed procedure was as follows: The solution (1 ml.) was added to the hydrazine reagent (2 ml.), left for 2 hr., filtered, extracted with chloroform (5 ml.; 1 × 1 ml.), and centrifuged. An aliquot part (2 ml.) of the aqueous layer (now 0·750M in perchloric acid) was added to a solution (2 ml.) 0·667M in disodium hydrogen phosphate and 0·50M in sodium hydroxide, cooled, filtered, extracted with chloroform (5 ml.; 2 × 2 ml.), and centrifuged. The optical density (327 mμ; 1 cm. silica cells) was measured in a Unicam spectrophotometer, with the phosphate solution as control. Using known amounts of pyridoxamine dihydrochloride in 0·01M-pyridoxal this method gave ε at 327 mμ = 8260. It is important to control the pH of the final solution within 0·1—0·2 unit, since the spectrum of pyridoxamine varies markedly with small changes in pH. The composition of the phosphate solution, given above, has been calculated to

neutralise the perchloric acid in the hydrazine reagent and to give pH 7·0. When the pH of the test solution differed significantly from this value, the acidity of the hydrazine reagent was varied accordingly. Estimation of the pyridoxamine in the experiment described above (under estimation of pyruvate) gave:

Time (min.)	0	45	90	135	180
Optical density (327 mμ)	0·090	0·438	0·660	0·920	1·130

The rate of change of optical density is, therefore, 0·0058 unit min.$^{-1}$: hence the initial reaction rate is $0{\cdot}0058 \times 6/(600 \times 8260) = 7{\cdot}02 \times 10^{-8}$ mole (of pyridoxamine) l.$^{-1}$ sec.$^{-1}$. The final solutions obtained on chromatography and on examination of the whole spectrum showed the presence of pyridoxamine only.

Kinetic Results.—Table 1 gives initial reaction velocities (10^7v, in mole l.$^{-1}$ sec.$^{-1}$) for the reaction between pyridoxamine and pyruvate. [B] and [K] refer to buffer and pyruvate

TABLE 1. *Reaction of* 0·02M-*pyridoxamine and pyruvate at* 25°.

(*a*) pH 10·0; $\mu = 0{\cdot}715$; B = *NN*-dimethylglycine (0·10M).

[K]	0·05	0·10	0·15	0·20	0·30	0·40	0·50	0·15 [a]	0·15 [b]
10^7v	0·84	1·37	1·55	1·74	2·06	2·15	2·36	1·08	2·04

(*b*) pH 9·8; B = CO_3^{2-}–HCO_3^- (0·20M).

[K]	0·02	0·04	0·08	0·15	0·20
10^7v	0·20	0·35	0·52	0·79	0·93

(*c*) pH 9·0; B = *NN*-dimethylglycine (0·10M).

[K] (M)	0·025	0·05	0·10	0·15	0·20	0·30	0·40	0·50	0·025 [b]	0·025
10^7v	0·86	1·49	2·32	2·86	3·11	3·51	3·73	4·05	0·61 [d]	0·86 [d]
[K] (M)	0·025 [c]	0·15 [b]	0·15	0·15 [c]	0·40 [c]	0·40	0·40 [c]	0·15 [b,g]	0·15 [g]	0·15 [a,g]
10^7v	1·31 [d]	1·73 [e]	2·86 [e]	5·10 [e]	2·34 [f]	3·73	5·25 [f]	2·32	3·90	7·18

(*d*) pH 8·0; B = triethanolamine (0·10M); $\mu = 0{\cdot}58$

[K] (M)	0·10	0·15	0·15 [e]	0·15 [c]	0·15 [b,h]	0·15 [h]	0·15 [c,h]
10^7v	0·97	1·10	1·21	1·02	1·32 [i]	1·77 [i]	2·25 [i]
[K] (M)	0·20	0·30	0·40	0·50	0·50 [b]	0·50 [c]	
10^7v	1·32	1·53	1·69	1·75	1·63	1·91	

(*e*) pH 7·0; B = phosphate (0·20M).

[K] (M)	0·02	0·08	0·16	0·24	0·32	0·15 [b]	0·15 [c]	0·15
10^7v	0·134	0·434	0·85	1·29	1·68	0·56	0·74	0·93

(*f*) (i) pH 8·0; B = triethanolamine (0·10M); [K] = 0·15M.

μ	0·23	0·40	0·58
10^7v	1·10	1·17	1·21

(ii) pH 9·0; B = *NN*-dimethylglycine (0·10M); [K] = 0·05M.

μ	0·10	0·60
10^7v	1·50	1·53

[a] [B] = 0·20M. [b] [B] = 0·05M. [c] [B] = 0·15M. [d] $\mu = 0{\cdot}23$. [e] $\mu = 0{\cdot}42$. $\mu = 0{\cdot}61$. [g] B = *NN*-Dimethylethanolamine. [h] B = *N*-Methyldiethanolamine. [i] $\mu = 0{\cdot}30$.

concentrations respectively. The pH values varied among individual runs by not more than ±0·04 unit from the stated value.

Table 2 gives initial reaction velocities (v_p,v_k = production of pyridoxamine and of pyruvate, respectively, in 10^{-8} mole l.$^{-1}$ sec.$^{-1}$), for the reaction between 0·01M-pyridoxal and alanine at 25° and pH 10, buffered by alanine ([A] = concentration of alanine).

TABLE 2. *Reactions of pyridoxal and alanine at* pH 10·0 *and* 25°.

[A]	10^8v_p	10^8v_k	v_p/v_k	[A]	10^8v_p	10^8v_k	v_p/v_k
0·10	4·05	2·10	1·93	0·60	13·7	7·20	1·90
0·20	7·01	3·55	1·97	0·80	—	7·56	—
0·40	—	5·02	—				

The apparent stoicheiometry of the reaction was determined at a series of times in an experiment at pH 10·0 and 25° with 0·01M-pyridoxal and 0·60M-alanine. In the following

Table, R is the proportion of total reaction at time t (based on pyridoxamine production), and [P] and [K] are the corresponding concentrations of pyridoxamine and pyruvate:

t (min.) ...	30	90	150	210	270	330	390	450
R	0·022	0·071	0·110	0·148	0·170	0·185	0·216	0·228
[P]/[K] ...	1·83	1·99	1·97	2·20	2·01	2·00	2·02	2·00

Table 3 shows the effects of added metal cations [M] on the reaction between 0·05M-pyruvate and 0·02M-pyridoxamine.

TABLE 3. *Effect of added cations.*

pH	M	10^7v	Increase due to M
10·00	Cu^{II}, 0·016M	*ca.* 200	*ca.* 100
7·00	Ca^{II}, 0·001M	1·52	nil
7·00	Mg^{II}, 0·001M	1·53	nil

Spectrophotometric Experiments.—The spectra of solutions containing pyridoxamine (*ca.* 10^{-3}M), potassium pyruvate (0·05—0·40M), suitable buffer (0·05M), and sodium chloride (to μ 0·50) were measured against control solutions containing all components except pyridoxamine, over the range 270—340 mμ in 0·1 cm. cells at 25° in a Unicam photoelectric SP 500 spectrophotometer. Where necessary the pH of each solution was adjusted to the desired value by addition of acid or base. The presence of potassium pyruvate reduces the absorption of pyridoxamine at 320 mμ and a new maximum appears at *ca.* 285 mμ. The changes were, within the limits of our observation, immediate and the spectra produced were stable for the period required for the examination, transamination being minimised by use of relatively dilute buffer. At all pH values investigated, sharp isosbestic points were observed (at 298, 294, 293·5, 292·8, 292 and 292·8 mμ for pH 6·70, 7·51, 8·03, 8·49, 9·55, and 10·55 respectively), showing that the spectral changes are associated with a single equilibrium.

The apparent equilibrium constant for the reaction:

$$\text{Pyridoxamine} + \text{Pyruvate} \rightleftharpoons S_2; \quad K_e = [S_2]/[P][K]$$

where S_2 is the product of reaction irrespective of structure and the concentrations are sums of all the possible ionic forms for each species, is, under conditions where pyruvate is always in large excess, given by the equation:

$$\frac{1}{(D_0 - D_i)} = \frac{1}{K_e[K]} \cdot \{\varepsilon_p[P]_0 - \varepsilon_s[P]_0\}^{-1} + \{\varepsilon_p[P]_0 - \varepsilon_s[P]_0\}^{-1} \quad . \quad . \quad (1)$$

where $[P]_0$ is the initial concentration (including all ionic species) of pyridoxamine, ε_p and ε_s are the molar extinction coefficients, at some particular wavelength, of pyridoxamine and of S_2, respectively, D_0 is the optical density at that wavelength of pyridoxamine alone ([K] = 0), and D_i is the optical density due to pyridoxamine and S_2 at a pyruvate concentration [K]. For the results at each wavelength a plot of $(D_0 - D_i)^{-1}$ against $[K]^{-1}$ should be a straight line, whence K_e is found by dividing the intercept by the slope. The results gave good straight lines at all wavelengths for each pH studied, indicating that the formation of S_2, consistently with the above formulation, involves equimolar amounts of reactants.

The spectra obtained in a typical experiment are shown in Fig. 1. Table 4 gives the values of K_e (l. mole^{-1}) obtained at a series of wavelengths.

TABLE 4. *Equilibrium constant for formation of* S_2 *from pyridoxamine and pyruvate at* pH 8·49.

λ (mμ) ...	270	272·5	275	277·5	280	282·5	285	287·5	290	297·5	300	302·5	305
K_e	6·3	6·1	6·2	6·0	6·5	6·2	6·6	6·3	6·1	6·6	6·6	6·3	6·5
λ (mμ) ...	307·5	310	312·5	315	317·5	320	322·5	325	327·5	330	332·5	335	
K_e	6·4	6·6	6·6	6·6	6·6	6·4	6·5	6·2	6·1	6·2	6·5	6·6	

The precision with which K_e is found increases as $(D_0 - D_i)$ increases. In finding the mean value of K_e for each set of results the values at individual wavelengths have, therefore, been weighted approximately in proportion to the value of $(D_0 - D_i)$ observed at the wavelength. The weighted mean values of K_e so obtained at a series of pH values are given in Table 5.

TABLE 5. *Variation of* K_e *with* pH.

pH	6·70 [1]	7·51 [1]	8·03 [2]	8·49 [3]	9·55 [4]	10·55 [4]
K_e	1·15	2·20	4·94	6·40	9·1	8·9

Buffers: [1] Phosphate. [2] Triethanolamine. [3] *N*-Methyldiethanolamine. [4] *NN*-Dimethylglycine.

For each set of results, values of ε_s at each wavelength can be calculated for each pyruvate concentration from the equation

$$\varepsilon_s = [P_0]^{-1} \cdot \{D_i + (D_i - D_0)\}/K_e[K]. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Good agreement among the values for various pyruvate concentrations was obtained, the maximum difference between individual values being usually less than 5%. The spectra of

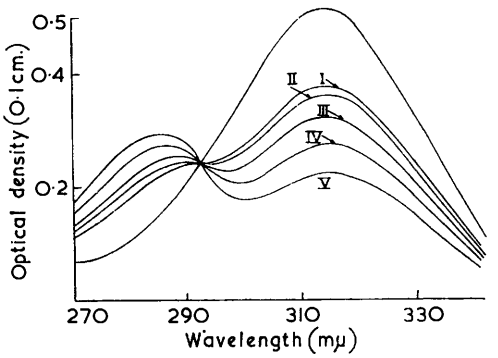


FIG. 1. *Spectra of mixtures of pyruvate and* 8·176 × 10^{-4}M-*pyridoxamine at* pH 8·49, *in* 0·05M-*diethanolamine, made up to* μ 0·50 *with sodium chloride.*

Pyruvate concn.: I 0·0715, II 0·0886, III 0·124, IV 0·198, V 0·314M.

FIG. 2.

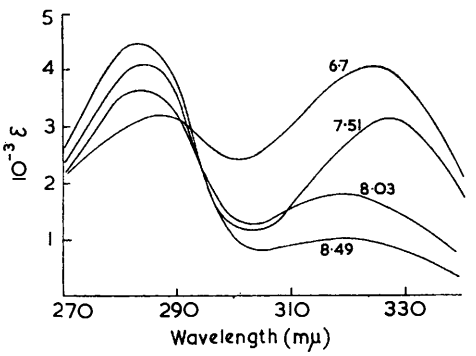
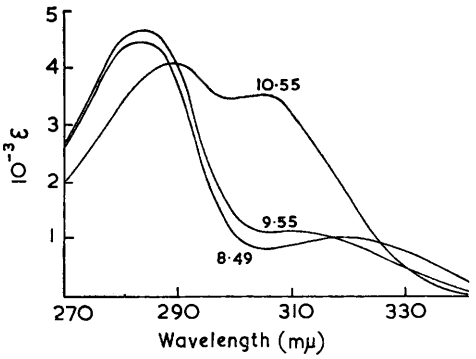


FIG. 3.



FIGS. 2 and 3. *Spectra of the intermediate* S_2 *at the* pH's *marked on the curves.*

S_2 obtained in this way are shown in Figs. 2 and 3 (over the ranges pH 6·7—8·5 and 8·5—10·5 respectively).

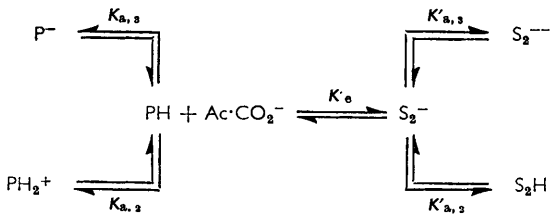
DISCUSSION

We have found that, in the range pH 6·7—10·5, pyridoxamine and pyruvate * react to form a new substance (S_2). The reaction, which is conveniently observed spectroscopically, is fast and reversible and involves equimolar amounts of reactants. Subsequent formation of pyridoxal and alanine (*i.e.*, transamination) is relatively slow and by appropriate choice of conditions the formation of S_2 can be studied without complication.

Under our conditions pyridoxamine exists in three dissociation states (PH, P^-, PH_2^+; $pK_{a,2}$ and $pK_{a,3}$ = 8·08 and 10·33 respectively). If we suppose that S_2 likewise exists

* Under all conditions studied by us, pyruvic acid is fully dissociated. The word pyruvate is used, therefore, to mean the conjugate base of pyruvic acid, *i.e.*, $Ac{\cdot}CO_2^-$.

in three states and that the carboxyl group derived from pyruvate is fully ionised, the equilibria may be formulated as:



K'_e should be independent of pH (except below the pH where protonation of the pyruvate carboxyl may be important) and is related to K_e by the expression:

$$K_e = K'_e \cdot \frac{1 + \frac{K'_{a,3}}{[H^+]} + \frac{[H^+]}{K'_{a,2}}}{1 + \frac{K_{a,3}}{[H^+]} + \frac{[H^+]}{K_{a,2}}} = K'_e\left(\frac{\alpha}{\beta}\right). \quad (3)$$

K_e is virtually independent of pH over the range *ca.* 9·0—10·5 (Table 5). In this region, however, pyridoxamine loses a proton (PH ⟶ P⁻; $pK_{a,3} = 10{\cdot}33$). Inspection of

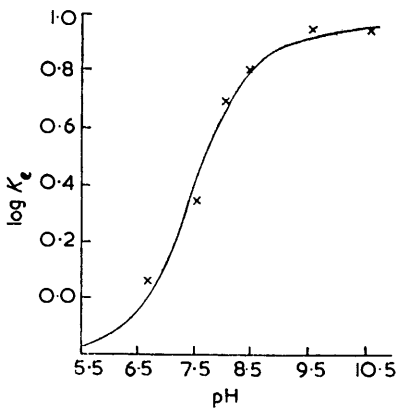


FIG. 4. *Plot of* log K_e *against* pH ($pK'_{a,2} = 6{\cdot}9$). × *Experimental values.*

equation (3) shows that these two facts are consistent only if $K'_{a,3} = K_{a,3}$. It remains to discover the value of $K'_{a,2}$. This must differ from $K_{a,2}$ since K_e decreases considerably below pH 9·0. Manipulation of equation (3) gives:

$$\text{d log } K_e/\text{d pH} = \text{d log } (\alpha/\beta)/\text{d pH}. \quad (4)$$

Since $K'_{a,2}$ is the only unknown in (α/β), trial substitution until the curve of log K_e against pH fits the curve log (α/β) against pH gives the required value. Fig. 4 shows the theoretical curve, constructed in this way, of log K_e against pH for $pK'_{a,2} = 6{\cdot}9$. The fit with the experimental points is good. It is considered that the value given is correct within $\pm 0{\cdot}1$ pK unit.

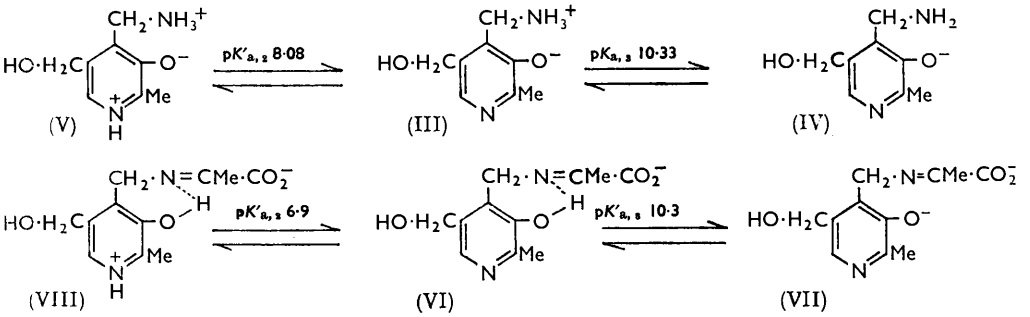
The following facts are consistent with the formulation of S_2^- as (II) (R = Me, carboxyl ionised); (*a*) its formation is rapid and reversible; (*b*) it is derived from equimolar amounts of reactants; and (*c*) at each pH progressive addition of pyruvate gives spectra showing a sharp isosbestic point. Convincing evidence that Schiff's bases are formed between pyridoxal and amino-acids comes from Metzler's work.[6] Consistently, in the present work, potassium α-4-pyridoxylideneaminopropionate has been prepared from ethanolic solutions of pyridoxal and the potassium salt of alanine. A similar experiment, starting with pyridoxamine and potassium pyruvate, gave only starting materials, pyridoxal and

alanine. However, immediate addition of nickel ions gave a complex, which from its analysis and properties, appears to be nickel di-(α-4-pyridoxyliminopropionate) [*i.e.*, $Ni(S_2)_2$]. Under certain conditions this complex isomerises to nickel di-(α-4-pyridoxylideneaminopropionate), which was independently prepared from an ethanolic solution containing pyridoxal, the potassium salt of alanine, and nickel ions.* These observations also support the view that the product from pyridoxamine and pyruvate is a Schiff's base.

The spectrum of pyridoxamine changes considerably with pH. Lunn and Morton [10] reported that the changes are not associated with a sharp isosbestic point. We have confirmed this. At pH 9·55, $\lambda_{max.} = 310$ mμ (ε 6850). As the pH decreases, $\lambda_{max.}$ shifts to longer wavelengths (326 mμ at pH 6·75). The extinction coefficient at first decreases and then increases (6250, 6720, 7130, and 7710 at pH 8·49, 8·03, 7·51, and 6·75, respectively). There is no isosbestic point. This phenomenon presumably arises because of equilibria between zwitterion and non-zwitterion forms of the formally neutral (PH) and of the protonated (PH_2^+) pyridoxamine species.[10,11] The spectra of the condensation product (S_2) also show no single isosbestic point. This is clearly shown for pH 8·5—10·5 in Fig. 3.† It may be supposed that the explanation is similar to that applied to pyridoxamine.

Based on the discussions given by Metzler and Snell,[11] by Metzler,[6] and by Lunn and Morton,[10] the following are arguments for assigning the structures annexed to the various forms (existing in the range pH 6·7—10·5) of pyridoxamine (III)—(V) and of the condensation product (VI)—(VIII).

(*a*) Progressive addition of ethanol to an aqueous solution of pyridoxamine at pH *ca.* 9·0 shifts the absorption peak at 305 mμ to shorter wavelength. In absolute ethanol, absorption occurs at 284 mμ (ε 4800): there is no sharp isosbestic point. Comparison with results for pyridoxine and related compounds [11] leads to the view that absorption at 284 mμ is associated with the uncharged neutral pyridoxamine molecule, and that the shift to longer wavelengths in the more polar solvent is due to a zwitterion species. Structure (III) is preferred to the alternative form (involving protonation of the ring-nitrogen atom) since the latter would be expected to absorb at *ca.* 325 mμ. Loss of a proton leads to structure (IV) unequivocally. Addition of a proton (pH 8·5—7·5) shifts the absorption to longer wavelength ($\lambda_{max.}$ 325 mμ); this, by analogy, is characteristic of protonation of the ring-nitrogen atom: hence, PH_2^+ has the structure (V).



(*b*) In alkaline solution, structure (VII) must represent the species S_2^{2-}. The strong absorption at 310 mμ (Fig. 3) is characteristic of such structures. Below pH 10 addition

* The structure of these complexes is, at present, unknown. It appears from the discussion of Ni^{II} complexes given by Gill and Nyholm [9] that the magnetic results available do not permit unambiguous choice between a tetrahedral and an octahedral structure to be made. This aspect is being investigated.

† Since the spectrum change on addition of pyruvate to pyridoxamine is so small in the more acid region, the divergence from an isosbestic point in the range pH 6·7—8·5 (Fig. 2) may be due to experimental error. However, the point remains: over the whole region there is no single isosbestic point.

[9] Gill and Nyholm, *J.*, 1959, 3997.
[10] Lunn and Morton, *Analyst*, 1952, **77**, 718.
[11] Metzler and Snell, *J. Amer. Chem. Soc.*, 1955, **77**, 2432.

of a proton occurs and the main absorption shifts to 284 mμ. This indicates that S_2^- may be represented by formula (VI). This structure, which is presumably in equilibrium with its zwitterion isomer (some absorption at 325 mμ being still present), is similar to that proposed by Metzler [6] for the imine (IX; R = Pr^i) formed from pyridoxal and valine. Hydrogen-bonding between the phenolic group and the imine-nitrogen atom decreases the acidity of the former in both cases [p$K_{a,3}$ = 10·3; cf. 10·5 for (IX) and 8·2 for the phenolic group of the neutral, non-zwitterion form of 3-hydroxypyridine [11]]. At pH's between 6·5 and 7·5 the addition of a proton (giving S_2H) is accompanied by an increase in absorption at 325 mμ. Structure (VIII) is, therefore, indicated for S_2H. The pyridine-nitrogen atom is more basic in compound (VI) than in the homologue (IX) (pK 6·9 and 5·9,[6] respectively). This is not unreasonable, since in the latter the ring-nitrogen atom is part of a more extended conjugated system.

(IX)

The structures suggested are, in some measure, tentative and, in any case, represent only the predominant forms under particular conditions. They provide, however, a reasonable interpretation of the spectra and of the dissociation constants.

Solutions of pyridoxamine and pyruvate at 25° slowly produce pyridoxal and alanine. Both products have been identified chromatographically. A kinetic study, based on analysis of pyridoxal, has been made. The initial rate of production of pyridoxal (v) is not appreciably affected by changes in ionic strength (sodium chloride), but depends markedly, at fixed substrate concentrations, on pH and on the nature and concentration of the buffer. At pH >8·0, under particular conditions of pH and buffer concentration, the reaction is of the first order with respect to pyruvate at low concentrations, and tends to zero-order at high concentrations (see Fig. 5 for the results at pH 9·0). This behaviour, which is analogous to that shown by simple enzyme systems, indicates that reaction involves an intermediate. If we assume, for the moment, that the intermediate is S_2, then the reaction scheme becomes:

$$\text{Pyridoxamine} + \text{Pyruvate} \rightleftharpoons S_2 \xrightarrow{k_2} \text{Products}$$

and the appropriate rate equation (in terms of initial reaction rate), is

$$\frac{1}{v} = \frac{1}{[\mathrm{K}]_0}\left\{\frac{1}{K_e k_2 [\mathrm{P}]_0} + \frac{1}{k_2}\right\} + \frac{1}{k_2[\mathrm{P}]_0}, \qquad (5)$$

where K_e is as previously defined (*i.e.*, in concentration terms which include all the ionic states of S_2 and P), k_2 is the first-order rate coefficient for the process by which S_2 passes into products,* and $[\mathrm{P}]_0$ and $[\mathrm{K}]_0$ are the initial concentrations of substrates.

Equation (5) requires that a reciprocal plot of v against $[\mathrm{K}]_0$ (for fixed $[\mathrm{P}]_0$) should be a straight line; whence K_e is given by

$$K_e = \{(S/I) - [\mathrm{P}]_0\}^{-1}, \qquad (6)$$

where S and I are the slope and intercept respectively. Each set of results, plotted in this way, gave a good straight line. At pH 9·0 (0·10M-*NN*-dimethylglycine buffer), the results (Fig. 5) led to the values K_e = 9·8 (cf. 9·0 for the interpolated spectroscopic value), $k_2 = 2{\cdot}56 \times 10^{-5}$ sec.$^{-1}$.

The value of v for fixed substrate concentrations depends, as stated above, on the nature and concentration of the buffer used. Analysis, by equations (5) and (6), of results obtained at the same pH but with different buffer concentrations gives the same value of K_e but different values of k_2. Hence, as might have been expected, the buffer influences

* If S_2 reacts in all its ionic states, then k_2 is, for fixed conditions, the sum of the individual coefficients. The scheme does not imply that S_2 passes directly into products but only that the rate-determining step involves S_2.

the rate of reaction of S_2 and not its equilibrium concentration.* Let us assume that k_2 may be expressed in the form:

$$k_2 = (k_2^0 + k_2'[B]), \quad (7)$$

where k_2^0 is the rate coefficient for reaction of S_2 in the absence of buffer and k_2' is the catalytic constant for the particular buffer of concentration [B]. Since $v = k_2[S_2]$, we have, at fixed substrate concentrations:

$$v \propto (k_2^0 + k_2'[B]). \quad (8)$$

The results obtained at pH 9 conform with this equation (Fig. 6). The value of v increases linearly with [B] for each pyruvate concentration. With two different buffers (*NN*-dimethylglycine and *NN*-dimethylethanolamine), the lines intersect the axis at the same

FIG. 5. *Kinetics of the reaction between pyridoxamine and pyruvate* (K) *at* pH 9·0.

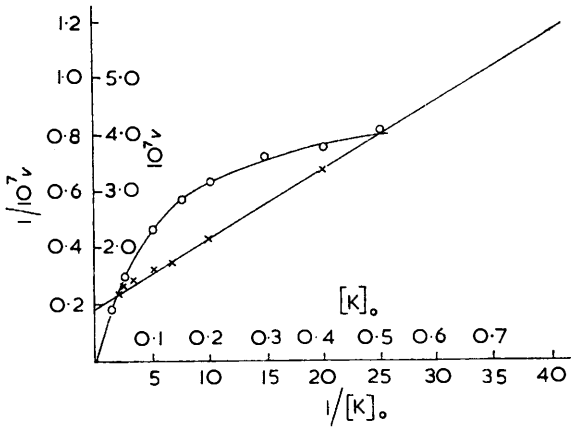
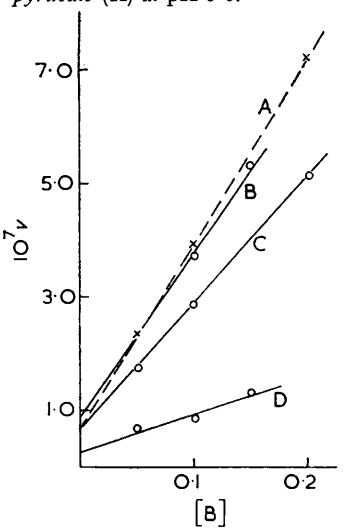


FIG. 6. *Buffer catalysis in the reaction between pyridoxamine and pyruvate* (K) *at* pH 9·0.



A, NN-*Dimethylethanolamine and* $[K]_0$ 0·15M.
B—D, NN-*Dimethylglycine and* $[K]_0$ (B) 0·40M, (C) 0·15M, (D) 0·025M.

point; hence k_2^0 is independent of the buffer used. Elimination of k_2 between equations (7) and (5) gives:

$$(v_0/v)_{K_0} = k_2^0/(k_2^0 + k_2'[B]), \quad (9)$$

where v and v_0 are the velocities at buffer concentration [B] and zero, respectively, and refer to a pyruvate concentration $[K]_0$. At a fixed value of [B], the velocity ratio (of equation 9) was found to be independent of $[K]_0$ at each pH studied, hence k_2^0 is independent of $[K]_0$. For example, at pH 9·0 and [B] = 0·10M, the following values were obtained:

$(v_0/v)_{K_0}$	0·23	0·22	0·23
$[K]_0$	0·025	0·15	0·40

By the use of equations 5—9 the kinetic results for each pH were analysed; the collected results are given in Table 6. At pH 6·85 the analysis is insensitive, since the

* To avoid complications from imine-forming reactions, none of the buffers used contained primary amino-groups.

reciprocal plot $\frac{1}{v}/\frac{1}{[K]_0}$ passes close to the origin (*i.e.*, the reaction is nearly of the first order in pyruvate over the whole range of pyruvate concentration). The values given for K and k_2^0 at this pH are, therefore, approximate only.

TABLE 6. *Kinetic parameters for reaction of pyridoxamine and pyruvate at 25°.*

pH	Buffer	K_e *	10^5k_2 (sec.$^{-1}$)	$10^4k_2'$ (l. mole^{-1} sec.$^{-1}$)
6·85	Phosphate	~1·0 (1·15)	>1·0	—
8·0	Triethanolamine	8·2 (4·9)	0·95	0·18
9·0	N,N-dimethylglycine	9·8 (8·3)	0·95	1·98
9·8	Carbonate-bicarbonate	9·9 (9·1)	—	—
10·0	N,N-dimethylglycine	8·9 (9·0)	0·59	0·92

* Values in parentheses are interpolated from the spectroscopic results.

The values of K_e in Table 6 are in good agreement, considering the possible sources of error, with the more accurate spectroscopic values. They support the view that the rapidly formed intermediate which is observed spectroscopically (S_2; α-4-pyridoxylimino-propionate) is also involved in the reaction which produces pyridoxal.

Kinetic results obtained at pH 10·0 on the reverse process (*i.e.*, formation of pyridoxamine and pyruvate from pyridoxal and alanine) indicated that this reaction also involves an intermediate (K_e = 1·98, 1·87 by analysis of pyruvate and pyridoxamine, respectively). By analogy and consistently with Metzler's spectroscopic studies,[6] this intermediate is isomeric with S_2 (*i.e.*, it is α-4-pyridoxylideneaminopropionic acid, S_1). The rate-determining step for the overall reaction is then, on this view, the prototropic rearrangement $S_2 \rightleftharpoons S_1$. The mechanism of this rearrangement has not been established but the following points are important: (*a*) The rearrangement is general-acid catalysed. This follows since, whereas k_2^0 does not change in the pH range 10·0—9·0, k_2' (with *NN*-dimethylglycine as buffer) increases in approximately the same proportion as the conjugate acid of the buffer (2·2, 2·5, respectively). (*b*) In the absence of buffer the rate (k_2^0) increases somewhat below pH 9. This indicates that the species (VIII) may isomerise (perhaps by initial proton transfer from the ring-nitrogen to the ethylenic carbon) more easily than the species (VI) and (VII).

A few experiments were carried out in the presence of added metal cations. At pH 7·0, Mg^{II} and Ca^{II} (0·001M) had no effect on the initial rate. However, Cu^{II} (0·016M; at pH 10·0) had a powerful catalytic effect. *A priori*, catalytic effects of metal cations could arise in two ways: the concentrations of the intermediates (S_2 and S_1) could be effectively increased by complex formation and/or isomerisation in the complexes could be easier than in the intermediates themselves. In the experiment with added Cu^{II} the second possibility appears to operate since the conditions were such that the formation of S_2 was *ca.* 85% complete in the absence of the added cation. This aspect is being further investigated.

The reactions discussed above are not the only ones which occur in this system. It was found that at pH 10·0 the reaction of pyridoxal and alanine produces pyridoxamine and pyruvate in the molar ratio 2 : 1, and not in equimolar amounts as required by equation (2): the reason is unknown. The spectroscopic results show that the reversible formation of S_2 undoubtedly involves equimolar amounts of substrate. Metzler's work,[6] and the fact that the kinetic value of K_e is the same irrespective of whether the reaction is followed by production of pyridoxamine of pyruvate, show that the same is true of S_1. Presumably, therefore, the isomerisation $S_1 \longrightarrow S_2$ is accompanied by another reaction which ultimately leads to pyridoxamine but not to pyruvic acid. Snell and his co-workers [1] have indeed shown that other reactions (*e.g.*, decarboxylation) occur in addition to transamination when mixtures of pyridoxal and amino-acids are heated. However, side reactions of this kind do not appear to explain the integral relation between the amounts of pyridoxamine and pyruvate actually found. Whether the isomerisation $S_2 \longrightarrow S_1$ is also accompanied

by another reaction is at present unknown. Both the expected products have been identified chromatographically, but because of the difficulty of determining small amounts of alanine in the presence of the other components, the molar ratio in which they are formed has not been established.*

The relevance of the above result to the problem of enzymic transamination is clear. If, as required by the Braunstein–Snell hypothesis, the overall reaction involves stepwise transamination of pyridoxal and pyridoxamine, the primary function of the protein must be to catalyse the prototropic rearrangement of the imine intermediates.

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* The overall stoicheiometry cannot be determined by allowing the reactions to proceed to high conversion because, under these conditions, complications arise from condensation of pyridoxal and pyridoxamine. The condensation product (presumably an imine) readily undergoes aerial oxidation at pH 11·0 to produce, apparently, 2 mol. of pyridoxal.